Ubiquitin Localization Changes as *C. elegans* Undergo Stress

by

Jacob Sanders

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Thesis Committee:

Dr. Lynn Boyd, Chair

Dr. Matt Elrod-Erickson

Dr. Jason Jessen

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listened. Chris Cannon, my best friend, I thank you for your constant support and friendship. Most importantly, I would like to thank my mother, Theresa Sanders, who has taught me how to persevere and given me the encouragement to chase my dreams. She has been the greatest role model and I strive to be like her every day.

ABSTRACT

Muscle tissue is constantly undergoing protein turnover and reacts quickly to stress. Organisms have developed various mechanisms to help deal with stresses induced by their environment or other factors. The ubiquitin-proteasome degradation pathway has been shown to aid in the removal of misfolded or damaged proteins. Ubiquitination participates in tagging unwanted proteins for degradation and in other important signaling pathways such as endocytosis, cell cycle, histone modifications, *etc*. Ubiquitin, a highly conserved 76 amino acid protein, is the focus of our studies. Using confocal microscopy, we analyzed the localization of ubiquitin in aging, salt stressed and nutrient deprived muscle cells of C*aenorhabditis elegans* in the hopes of understanding the role ubiquitin has during stress induced conditions. Utilizing a GFP::Ub fusion protein and the *unc-54* promoter, we have shown that ubiquitin is evenly distributed in unstressed muscle nuclei; however, muscle nuclei that undergo stress form nuclear foci. Our research shows that *C. elegans* exposed to high salt and nutrient deprivation induce foci formation within the muscle nuclei. We have shown that Ub foci also form as worms age. However, heat shock and cold shock do not cause the formation of nuclear foci. Furthermore, we have shown that intestinal cells also exhibit nuclear foci when exposed to stress, albeit at a slightly smaller frequency.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I:

INTRODUCTION

Cells are constantly experiencing changing environments. Cellular homeostasis can be affected by changes in diet, sickness, or exercise. During times of cellular unrest, numerous amounts of cellular processes occur to react to the altering environment. Various proteins regulate these reactions and can affect the amount of specific proteins that are present in each cell. Ubiquitin (Ub) is one of many regulatory proteins that is involved in the regulation of proteins during cellular unrest. Ub received its name due to its abundance across various cell types and exists in all eukaryotes; however, there are no known prokaryotes that contain the protein. Ub is a 76 amino acid protein and acts in almost all cellular pathways as a post translational modifier that can target a substrate for proteasomal degradation, protein trafficking, DNA repair, histone modification, and many other processes (Hershko and Ciechanover, 1992). These interactions take place through the attachment of Ub to a target substrate. This can be done by the attachment of a single Ub (monoubiquitination) or multiple Ub proteins (polyubiquitination) which form Ub chains. These chains are constructed with one of seven lysine residues that serve as acceptors of Ub. The lysine residues include: K6, K11, K27, K29, K33, K48, and K63 (Peng et al., 2003; Matsumoto et al., 2005). The chains that are established due to ubiquitination can trigger specific processes to occur which can change the dynamics of a particular cell. These lysine residues are dispersed around Ub and point in all directions allowing other Ubs to connect to each other to form chains utilizing isopeptide bonds.

Without the function of Ub and its implications within cells, organisms would not be able to survive and flourish within their changing environments.

Ubiquitin Pathway

Ub attaches to its substrates through the aid of a special class of enzymes. The enzymes, as well as Ub, are highly conserved amongst eukaryotes and necessary for proper ubiquitination. These enzymes are the E1 (activating enzyme), E2 (conjugating enzymes), and E3 (ligase enzymes). The enzymes act sequentially in attaching Ub to the substrate and are an integral component for the Ub pathway to function. The first step, powered by ATP, begins when the E1 enzyme forms a thioester bond with the C terminal glycine of Ub, thereby activating the pathway. After the thioester bond has been formed, Ub is then transferred to the E2. Once this transfer occurs, the E2 conjugating enzyme then forms a complex with the E3 ligase enzyme. The E2/E3 complex can allow for direct Ub transfer to the substrate or Ub can transfer from the E2 to the E3 and then subsequently attach to the substrate. There are typically very few E1s, a greater variety of E2 enzymes, however E3s are the most specific and cells may contain hundreds of varying E3s (Hershko and Ciechanover, 1992). E3s are specific for substrates and will only allow transfer of Ub to substrates that it recognizes (Figure 1).

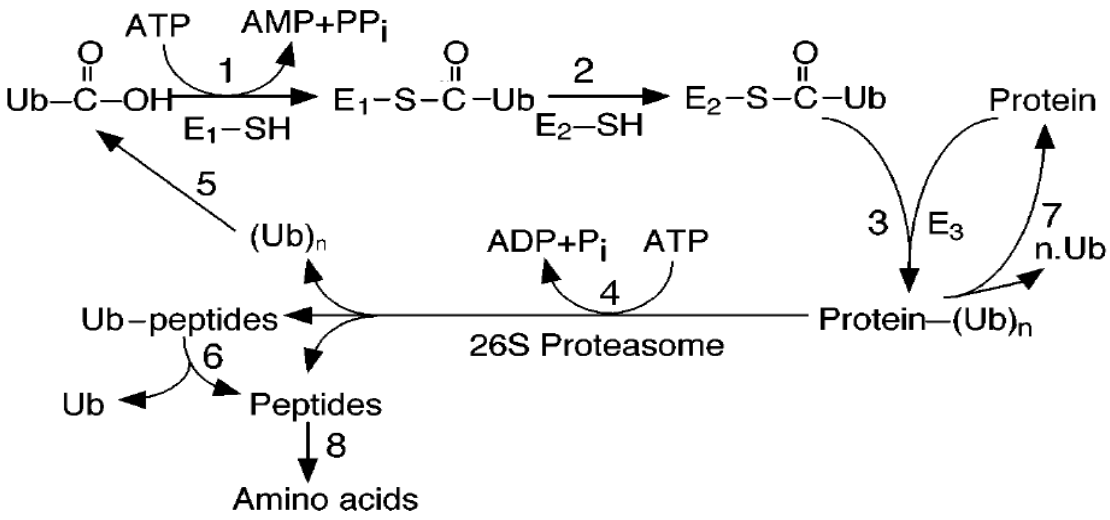

Figure 1. Schematic of Ubiquitin-Proteasome System

(Hershko and Ciechanover, 1998) Mechanism showing the process of Ub tagging a substrate for degradation.

Ubiquitin Chains

Ub chains determine what process the substrate will subsequently be involved in. Various studies have been conducted to determine the involvement of these chains in specific cellular processes (Adhikari and Chen, 2009). The orientation of lysine chains can determine what cellular process will occur. Many of the chains are poorly understood and very little substrate targets are known for them. Evidence suggests that all Ub chains, except K63, are sufficient to mediate proteasomal degradation (Xu et al., 2009).

Much of the recent literature has focused on understanding K48 chains. K48 is the most studied and best understood Ub chain. K48 linked Ub chains have been shown to tag the unwanted substrates which are subsequently recognized by the proteasome. The second major step in protein degradation by Ub is the recognition of the substrate by the proteasome. The proteasome is composed of two units; two 19S caps and a 20S core. The 20S can further be divided into two α rings and two β rings (Smith et al., 2007). These units work together to aid in the process of protein degradation. Once a protein is recognized it enters through the 19S cap and into the 20S core. After entering the core the β rings break down the proteins into short peptide fragments that exit through the second 19s cap. These peptide fragments can then be recycled. A protein must be tagged with at least four Ub proteins to be recognized by the proteasome (Sloper-Mould et al., 2001).

K63 Ub chains are the second most understood chain (Xu et al, 2009). K63 chains are implicated in several cellular processes. K63 chains are generally thought to operate proteasome-independent, but does have an effect on protein homeostasis. K63 chains have been shown to regulate protein homeostasis by promoting the formation of protein inclusions and inducing autophagy to remove them from the cell (Tan et al. ,2008). Proteins such as p62 have been shown to be selectively degraded through autophagy (Pankiv et al., 2007). In addition to clearing proteins through autophagy, K63 has a key role in the sorting and packing of protein contents into endosomes and lysosomes and their subsequent degradation. ESCRT (Endosomal Sorting Complex Required for Transport) machinery requires K63 ubiquitination to have proper packing. (Lauwers et al., 2009) This process was later determined to be more successful with K63 polyubiquitination, rather than monoubiquitination. (Ren and Hurley, 2010) Recent claims suggest that ESCRT is responsible for not allowing K63 chains to interact with the proteasome (Nathan et al., 2013). Other reports conclude that K63 may be responsible for Lewy body formation via the E3 ligase, parkin (Lim et al., 2005).

Recent evidence has supported the importance of K11 chains in cell cycle regulation (Jin et al., 2008). Studies from human, *Drosophila,* and *Xenopus* have indicated APC/C activation during mitosis causes an increase in K11 chains. (Williamson et al., 2009; Wu et al., 2010) K11 chains appear necessary for proper cell division in *Xenopus.* Knockdowns of K11 chains and APC/C result in

similar defects of cell division. The role for K11 in cell cycle regulation has yet to be properly defined. Recent discoveries suggest that all Ub chains have the capability of proteasomal degradation, however, more information is needed to understand the full breadth of K11 chains functions in cell cycle (Xu et al., 2009). Current literature hypothesizes that K11 chains have some responsibility in proper spindle formation (Williamson et al., 2009). K11 chains have also been implicated to have a role endoplasmic reticulum associate degradation (ERAD). Studies have indicated that ERAD machinery is ubiquitinated when it contains misfolded domains. K11 chains, along with K48 chains, are thought to have a key role in the regulation of ERAD domains (Adhikari and Chen, 2009).

The function of the remaining lysine chains remains elusive, however, few reports have indicated they contain specific roles in proteasomal degradation. K6, K27, and K48 chains have been linked to forming atypical chains to regulate gene silencing by modifying histones (Ben-Saadon et al., 2006). K6 chains have also been associated with DNA repair. K6 chains have been shown to form at DNA damage sites by being targeted by BRCA (Sobhian et al., 2007). K27 chains have also been implicated in DNA damage by ubiquitinating histones H2A and H2A.X, markers of DNA damage. (Gatti et al., 2015) K33 ubiquitination remains the most elusive of all Ub chains and not many reports are available to indicate specific functions.

Ubiquitin and Stress

Most intracellular proteins are degraded and regulated through the Ub-proteasome system (UPS) or lysosomal degradation (Kraft et al., 2008). The UPS is the primary degradation pathway for short lived and damaged proteins. Organisms can undergo stress due to many external factors that can cause proteins to be damaged or unfolded. The UPS acts directly in response to certain stresses that organisms encounter and aid in promoting degradation of damaged proteins. Current literature supports the significance of Ub in various cellular stress responses, such as: starvation, salt stress, heat shock, and cold shock (Finley et al., 1987; Jung et al. 2012)

Starvation is a common stressor that affects many organisms. In order for cells to survive short term starvation periods, mechanisms have evolved to provide a more efficient response. Autophagy has long been associated with starvation, but its relationship with Ub has been a recent discovery within the last decade (Kraft et al, 2008). Since then, selective autophagy during starvation has gained more attention. Ribosomes have been observed to be ubiquitinated and degraded through autophagy during nutrient deprivation. However, Ub has been implicated in the starvation stress response for decades (Finley et al., 1987). Furthermore, studies using rats have shown proteolytic activity due to starvation is regulated by the UPS (Garlick et al. 1998). Induced constitutive autophagy also requires Ub to clear out abnormal proteins that form (Komatsu et al., 2005). Atrophying muscles also have a decrease in the Ub

ligase, atrogin-1. When atrogin-1 formation was induced atrophy decreases in *in vitro* muscle cells (Sandri et al., 2004).

Much of the research on salt stress as it relates to Ub has occurred in *Arabidopsis* or other plant model systems. However, osmotic stress has shown to dramatically effect cellular processes occurring within cells. Similar to the abnormal proteins formed during starvation, large ribonucleicprotein foci were found to form due to extreme osmotic stress (Jud et al., 2008). The role of Ub and ribonucleicproteins remain unclear. In *Caenorhabditis elegans,* inhibition of DAF-16/FOXO transcription factor allows for a longer lifespan of worms grown in a hypertonic environment (Lamitina and Strange, 2005). Furthermore, hyperosmotic stress causes widespread protein damage which causes misfolded and damaged proteins to form (Choe and Strange, 2008; (Burkewitz et al., 2012). The ability for an organism to survive requires the degradation of these proteins which are typically degraded by the UPS.

Other stressors that occur frequently and can have a lasting effect on organisms are changes in temperatures. Cells must maintain proper temperature for normal processes to occur. Enzymes and cellular processes depend on specific conditions for their ability to perform their functions efficiently. Many organisms have adapted mechanisms to maintain proper temperature and adjust to changes in temperature. Transcription factors such as HSF-1 play a significant role in heat stress. When cells undergo heat stress HSF-1 is targeted to the nucleus as a negative feedback loop initiator.

Furthermore, localization of HSF-1 to the nucleus causes the formation of HSF-1 foci to form. Hul5 in *Saccharomyces cerevisiae* is an important E3 ligase that functions to maintain cell fitness by degrading short lived misfolded proteins as a result from heat shock (Fang et al., 2011). Less is known about the relationship between cold shock and Ub, but one study concluded that Ub is synthesized during cold stress (Muller-Taubenberger, 1988).

Ubiquitin and Disease

Ub has been a key focus when studying neurodegenerative diseases. Neurodegenerative diseases, such as, Alzheimer's, Huntington's, and Parkinson's disease all share an Ub interest (Lowe et al, 1988; Bennett et al., 2007). These diseases have had a major impact on the global scale and continue to be highly studied diseases. Ub has been an area of potential promise for therapeutic drugs.

Alzheimer's patients are characterized as having excess neurofibrillary tangles, senile plaques, and deposits of amyloid-β (Salon et al., 2000). Current literature supports that the UPS is defective in clearing these structures. Moreover, plaques and tangles contain a mutant form of Ub (UBB+1). UBB+1 and hyperphosphorylated tau are considered to be interacting, however, the cause of impaired UPS function has not yet been defined (Van Leeuwen et al., 1998; Tai et al., 2012).

Huntington's disease (HD) is an autosomal dominant neurodegenerative disease that is characterized by late onset of symptoms that affect cognitive behavior. HD is caused by a CAG repeat in the huntingtin gene. The repeat expansions can reach over 40 repeats and code for glutamine. The length of the polyglutamine repeat can determine how early the onset of the disease will occur (Andrew et al., 1993). One distinguishing factor that signifies HD is the formation of inclusion bodies that are heavily enriched in Ub. However, recent studies suggest that Ub is not responsible for the initial formation of the ploy glutamine aggregates, but rather the secondary growth (Skibinski and Boyd, 2012).

Parkinson's disease (PD) is characterized by neuronal degeneration of dopaminergic neurons found in the substania nigra pars compacta (SNc). Cell death in the SNc leads to the formation of Lewy bodies, which like other neurodegenerative diseases contain poorly degraded proteins (McNaught et al, 2003). The two major proteins found in Lewy bodies include parkin and α-synuclein. Evidence shows that parkin is responsible for ubiquitinating α-synuclein and the absence of parkin prevents the formation of Lewy bodies (Chung et al., 2001). Additionally, proteasomal degradation appears to have an impaired function in PD patients (McKnaught and Jenner, 2001). Parkin and Pink1 are responible for working together to ubiquitinate outer mitochindrial proteins which are selectively degraded by autophagy (Pickrell and Youle, 2014).

The Model System

The free living nematode, *Caenorhabditis elegans,* has played an important role in studying genetics, development, and neuroscience. *C. elegans* is a hemaprohditic worm that is found growing in dried and rotting fruit. *C. elegans* was chosen as a model organism because of its transparent nature. Its transperancy makes it a great organism for observing changes in any internal organs. It has the ability to produce 200-300 progeny within a few days. The life cycle consists of four larval stages and adulthood. The full lifespan of *C. elegans* is about 2-3 weeks. During this time, worms grow to adulthood within two days of hatching.*C. elegans* is a simple eukaryote that provides an inexpensive organism to study a variety of complex scientific issues.

Studies using *C. elegans* have greatly increased scientific knowledge and has provided a variety of tools that can now be implemented in other organisms. The discovery of green fluorescent protein (GFP) as a genetic marker have provided a great opportunity to study a multitude of proteins which can be easily observed in *C. elegans.* (Chalfie et al., 1994) RNA interference (RNAi) was another major disccovery that allows for the knockdown of proteins. (Fire et al., 1998) Together with these tools, the complete genome for *C. elegans* has been sequenced. Cell fate has also been determined for all 959 somatic cells that make up the hermaphroditic worm. *C. elegans* can be easily manipulated to study specific proteins. Since the beginning, various neuroscience questions have been answered by studying the neurons of *C.*

elegans. RNAi and GFP together have led to many discoveries that were proving difficult to answer.

Muscle proteins are constantly being degraded and rebuilt to maintain the stability of muscles. Ubiquitination in muscle fibers has been associated with many myopathies, particularly atrophying muscles (Minetti et al., 1996; Semino-Mora and Dalakas, 1998). Understanding the role of Ub in muscular diseases is important due to the constant muscle protein turnover. One particular muscular disease that affects humans is inclusion body myositis (IBM) which usually occurs in elderly adults and has been linked to the UPS system and the subsequent aggregates that form during muscle wasting (Askanas et al., 1992). If the UPS is playing a role in this disease it would be pertinent to understand how and where Ub localizes in aging worms and subsequently humans. Ub may also play a role in stress response; therefore, determining how different stressors may affect Ub is also important. As previously mentioned, Ub has been implicated to play a role in DNA repair, autophagy, and other pathways. Therefore, stressors such as starvation, salt, and other stressors can be used to understand the role Ub plays in muscle stress. Furthermore, determining how other tissues are affected by these stressors can also provide insight as to how tissues work together to provide a homeostatic environment.

Objectives/Significance

The goal of our studies is to establish where Ub localizes in the muscle and intestinal cells of stressed *Caenorhabditis elegans* adults. We would like to observe if different stressors will change Ub response within *C. elegans*. Once a pattern of Ub localization in starved, aged, and salt, heat shock, and cold shock stressed worms is established, it is important to determine what is being ubiquitinated during each stress and what Ub chain/chains are formed. Ultimately, we would like to have a better understanding of how Ub functions during the stress response of *C. elegans*. Ub has been associated with many forms of stress; however, the mechanism and Ub response to these stresses have not been thoroughly studied. It is important to understand the Ub stress response and localization during these events to determine what the role of Ub is in these events.

CHAPTER II:

MATERIALS AND METHODS

Strains

The wild type Bristol N2 nematode strain and the OP50 bacterial strain were obtained from the *Caenorhabditis* Genetics Center. LN133 [*rcIs16* P*unc-54*::GFP::Ub + *unc*-119; *unc*-119(ed3)], LN139 [*rcIs35* P*unc-54*::mCherry::Ub + *unc-119*; *unc-119*(ed3) LB11-3] and LN157 [P*unc-54*::GFP] were obtained from the Boyd Lab library. ERT261 [jyEx128[*vha*-6p::GFP::Ub cb-*unc-119*(+)];ttTi5605 II; *unc-119*(ed9)] was obtained from the lab of Emily Troemel.

C. elegans Maintenance

Worms were cultured using standard methods except in cases of stress. Worms were grown on nematode growth media (NGM) plates containing 51mM NaCl, 17 g/L agar, 2.5 g/L peptone, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 5mg/ml cholesterol, and 1 mM KPO_4. NGM plates were seeded with *Escherichia coli* OP50 strain to provide nourishment. OP50 is an uracil auxotroph that limits the growth of the bacteria on NGM plates for better visualization of worms. Transgenic strains were kept at 25°C and wild-type was kept 20°C.

Synchronization

Worms were synchronized using a 20% hypochlorite solution (25% NaOH, 20% Bleach, and 55% dH_2O). Worms were washed off plates using M9 buffer (42 mM Na_2HPO, 22 mM KH_2PO_4, 86 mM NaCl, and 1 mM $MgSO_4$ brought to 1 L with H_2O) and spun down at 300 X G for 1 minute. M9 buffer was aspirated off leaving the worm pellet. 15 mL of hypochlorite solution was then added to pellet

and gently mixed until the cuticle of a worm was observed breaking. After another spin down and aspiration of hypochlorite solution. The pellet was washed twice with M9 buffer and placed on unseeded NGM plates. Once the eggs hatched, L1's were placed on seeded NGM plates and grown to 3 day adults.

Starvation

For starvation experiments, worms were synchronized and grown on NGM plates utilizing a starvation recipe documented in once they reached the 3 day adult age (Seidal and Kimble, 2011). The recipe is similar to normal NGM plates, however, peptone is not included in the recipe to prevent bacterial growth. Starvation media contains 51mM NaCl, 25 g/L Agar, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 5 mg/ml cholesterol, and 1 mM KPO_4 which is then dispensed into petri dishes. Extra agar is added to balance out the media. Worms were then placed on the starvation plates for 12, 24, 36, and 48 hours at 25°C.

Salt Stress

For osmotic stress events, worms were synchronized and grown until they reached the 3 day adult age. 3 day adults were the placed on NGM plates with elevated NaCl levels as compared to 51mM NaCl in normal NGM plates. To ensure that worms would not experience stress except by salt stress, excess salt was added to obtain 100mM-600mM NGM plates. Other salt methods include placing worms in a desired NaCl concentration solution or pouring the solution on top of a NGM plate. Worms exchange gases and liquids through their pores

therefore having the increased NaCl in the media offers the best method for introducing increased NaCl levels. This method also provides similar living conditions for worms that are being grown on plates. Placing worms in liquid may also cause unwanted stress on muscle cells of *C. elegans*. Worms were grown on increased NaCl plates for 1, 3, 6, 12, and 24 hours and immediately imaged.

Heat and Cold Stress

For heat shock experiments, worms were grown and synchronized on seeded NGM plates until the 3 day adult stage was reached. Worms were then placed in an incubator at 35°C for 20 minutes or 1 hour and immediately imaged. Similarly, cold stress experimental worms were grown and synchronized on seeded NGM plates until they reached 3 day adults. However, worms were then placed at 4°C for 30 minutes or 1 hour. After incubation, worms were immediately imaged.

Imaging

For larval and adult aging experiments, worms were imaged using a Nikon Inverted Microscope. All aging experiments were imaged at 40X. For stress experiments, images were obtained on a Zeiss LSM 700 Confocal microscope. Images were obtained on a 60X oil objective. Worms were anesthetized with a 1:20 dilution of 250mg/ml tetramisole and M9 buffer and mounted onto a microscope slide with a 10% agarose pad.

CHAPTER III:

RESULTS

Ubiquitin Foci Are Observed in Muscle Tissue

Ub is a very abundant protein that is found in most eukaryotic tissues. To determine the localization of Ub in muscle cells of *C. elegans*, GFP (LN133) and mCherry (LN139) reporter tags were fused to Ub and expressed using an *unc-54* promoter. These fusion constructs exhibited a diffuse pattern in the cytoplasm and nucleus of muscle tissue in both larval and adult stages when viewed with confocal microscopy. Comparisons were made between larval and adult stages of the LN133 and LN139 strains to determine the localization of Ub. (Figure 2 and 3) The LN139 strain contained multiple cytoplasmic foci in adult stages, however, LN133 contained foci mostly in the larval stages. As LN133 grew in age, fewer foci were observed compared to its larval stages. For this reason, LN133 was used to continue further studies. Although LN133 contained less cytoplasmic foci, nuclear foci were observed more frequently among adults. (Figure 4) To determine how frequently Ub nuclear foci formed in adults, adult worms were grown to 1, 3, 7, and 10 days old to observe foci formation patterns. (Figure 5) After observing 120 nuclei per age, 1 day adults contained 13% of Ub nuclear foci. The occurrence of Ub foci in 3 day adults was less than 1%. The number of nuclei containing foci increased dramatically in 7 and 10 day adults and resulted in over 30%. It is likely that 1 day adults contained a small number of nuclear foci due to the transition from larvae to adult. The transition from L4 to young adult is crucial for egg development and many changes in morphology occur during this

period. It is possible that these developmental changes can cause stress on the muscle tissue that is growing and expanding which can lead to nuclear foci formation. The formation of nuclear foci in older adults is likely due to the breaking down of misfolded or old proteins that are present in worms that are approaching the end of the normal life cycle. Due to the low amount of nuclear foci in 3 day adults, this stage was chosen to continue further studies.

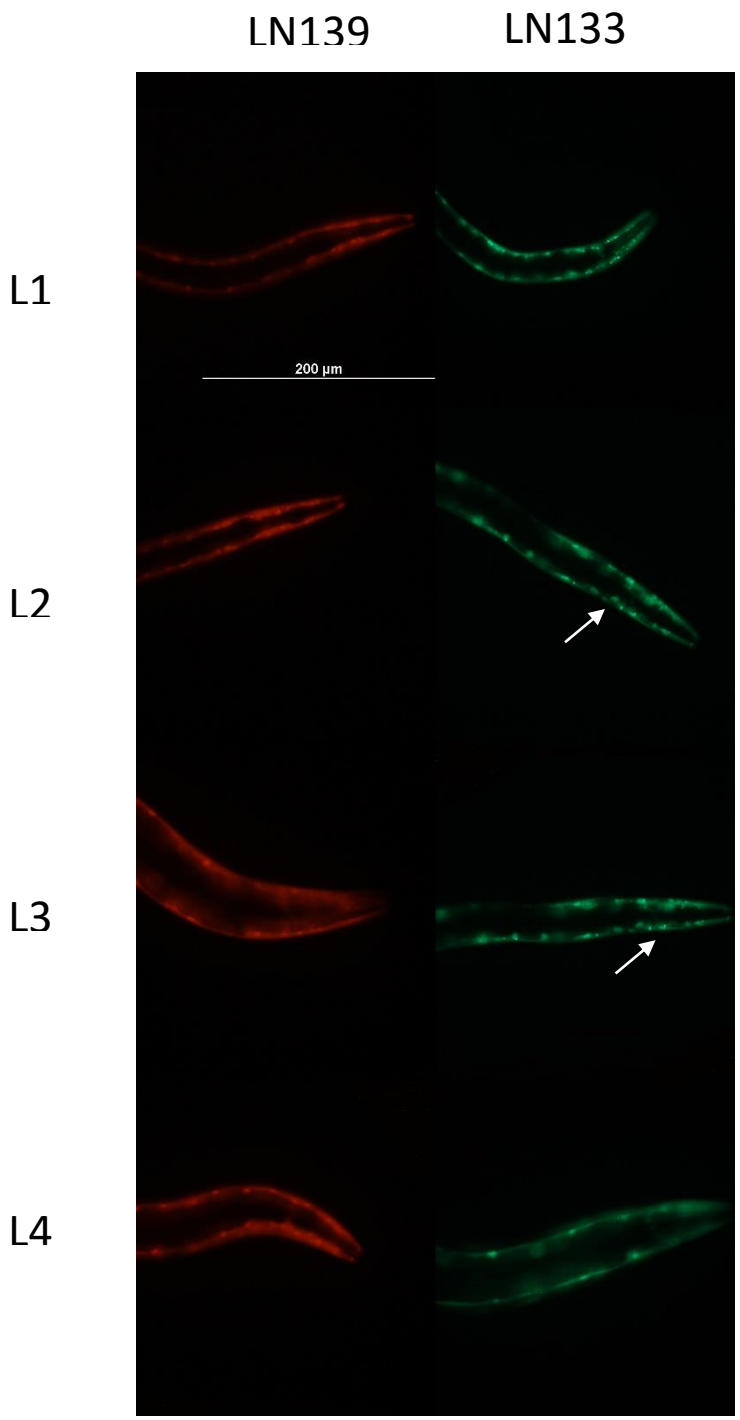

Figure 2. Ubiquitin Localization in Larval Stages. Larval comparison of ubiquitin using mCherry::Ub and GFP::Ub. L1, L2, L3, and L4 larvae were observed using a Nikon inverted microscope at 40X. Arrows indicate Ub foci forming in the cytoplasm.

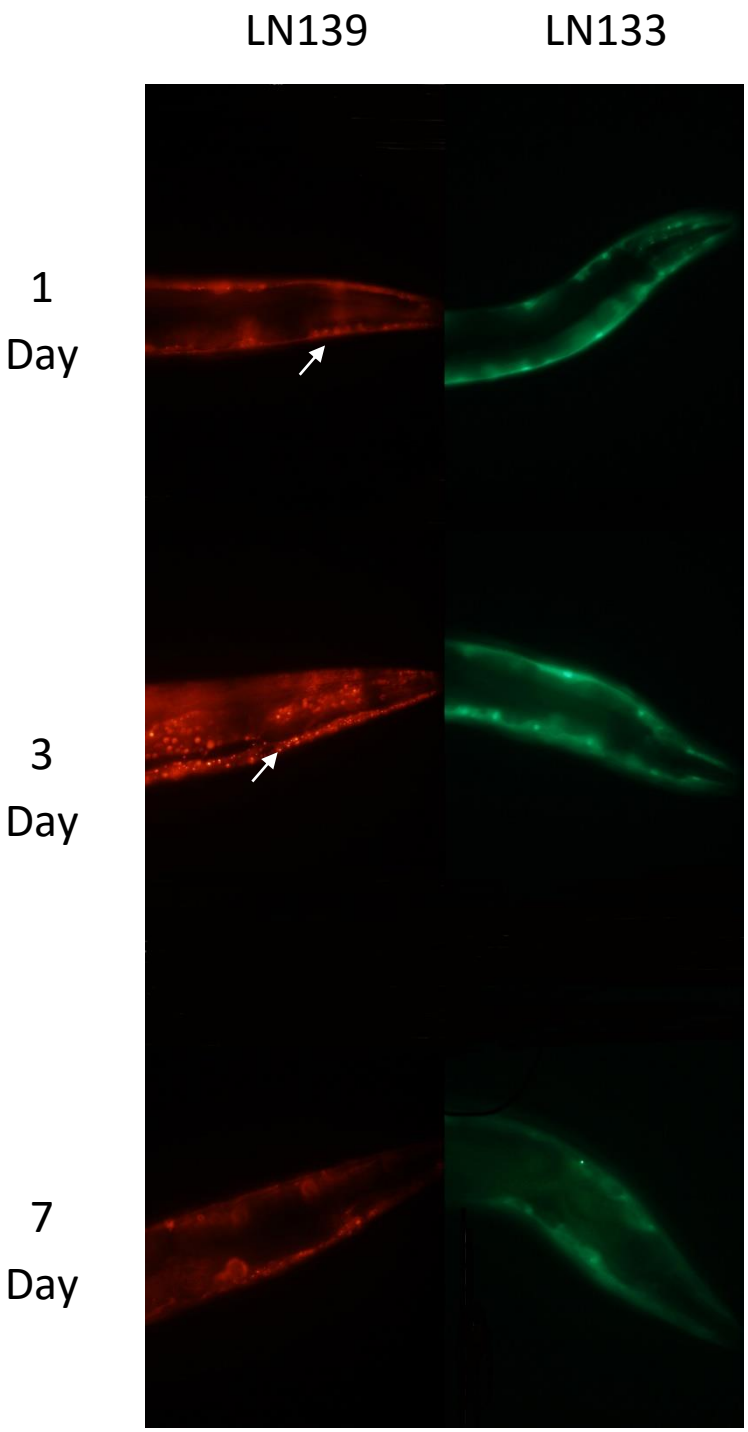

Figure 3. Ubiquitin Localization in Adults. Adult comparison of ubiquitin using mCherry::Ub and GFP::Ub. 1, 3, and 7 day adults were observed using a Nikon inverted microscope at 40X. Arrows indicate Ub foci forming in the cytoplasm.

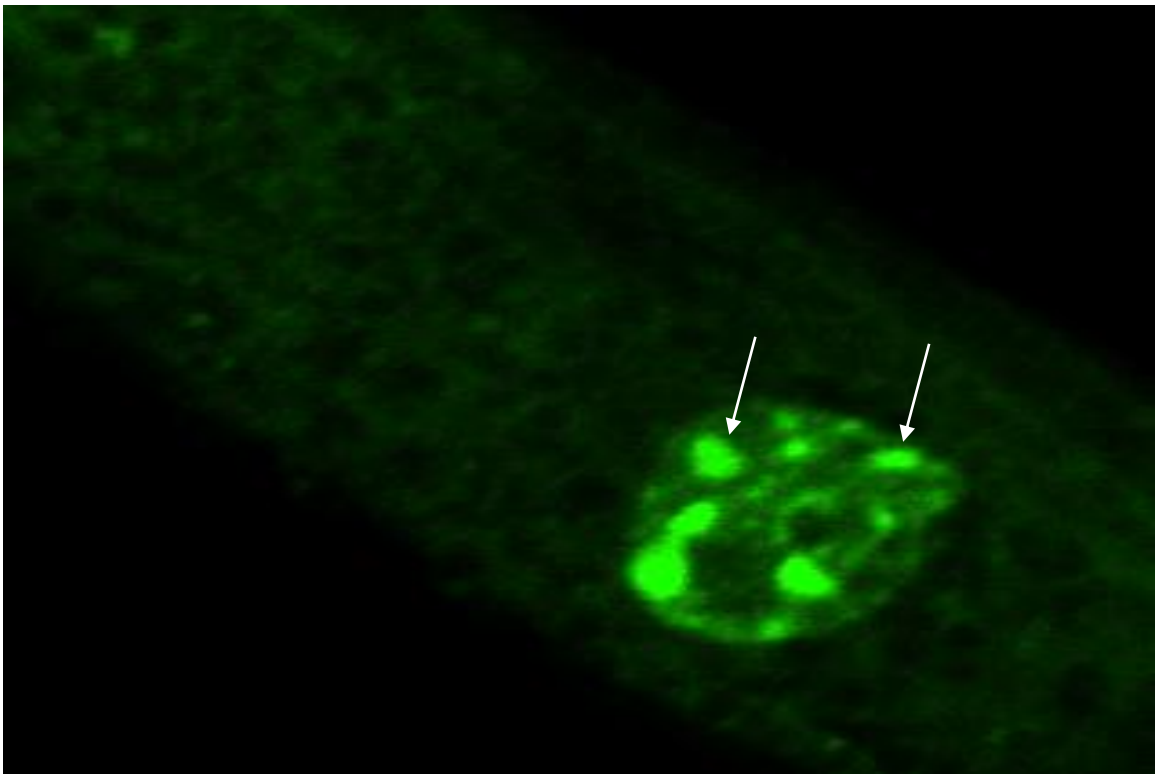

Figure 4. Ubiquitin Nuclear Foci in Aging Adult.
Nucleus of a 7 day adult with ubiquitin foci indicated by arrows.

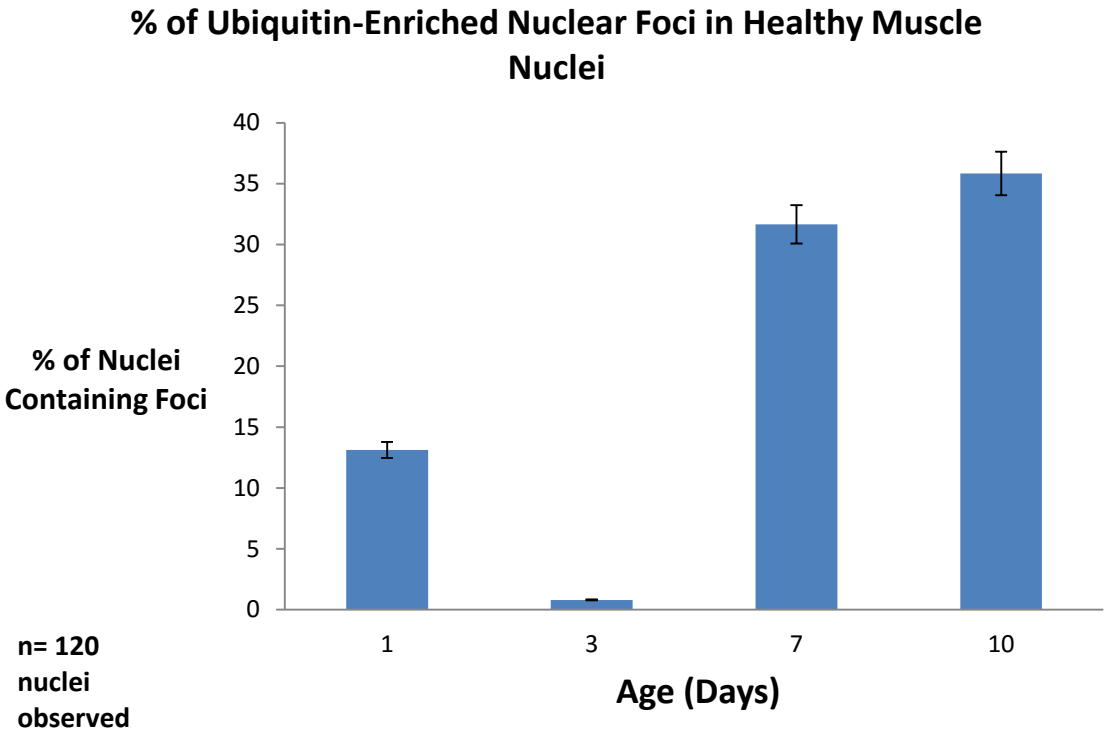

Figure 5. Foci are Observed in Aging Muscle Nuclei. Graph showing the percent of muscle nuclei that express foci in varying ages of *C. elegans*. 40 nuclei were observed in each of three separate experiments for a total of 120 nuclei observed. A total of 20 worms were observed over the course of the three experiments. Error bars represent standard error.

Nuclear Foci Observed in Starved and Salt Stressed Muscle Tissue

As cells age, the amount of misfolded proteins and senescent cells increase. These damaged and aged proteins are typically degraded after being tagged by Ub. In muscle cells, proteins are constantly being turned over. It is likely that as these cells age the amount of damaged cells increase. To establish if the formation of nuclear foci was an aging event or a stress event, worms were starved. Starvation has been shown to extend life span by signaling DAF-16 to reallocate resources. 3 day old LN133 adults were chosen for this experiment due to their low frequency of nuclear foci in the aging experiment. Worms were grown on starvation plates for 12, 24, 36, and 48 hours. At 12 hours there was minimal change, however, at 24 hours the amount of nuclei containing foci increased to 29%. The number of nuclei containing foci slowly dissipated as they reached 36 and 48 hours but were consistently higher than worms grown on plates with food. (Figure 6) Starvation did not seem to have an effect on worm morphology or behavior.

To test if other stressors could cause nuclear foci to form, worms were grown on NGM plates containing 100mM to 500mM NaCl for one hour. As compared to normal salt levels, worms began to show signs of nuclear foci at 400mM salt. 500mM salt plates were then chosen to determine the best concentration for future testing to determine possible substrates. 3 day adults were grown on 500mM salt plates for 1, 3, 6, 12, and 24 hours. Nuclear foci were observed at each time point, however, worms grown at 24 hours had a

high mortality rate. Salt stress had a much more dramatic effect on the worms when compared to aging, starved, and control worms. At 1 hour and 3 hour conditions 40% of nuclei contained Ub foci. At 6 hours, muscle nuclei containing Ub foci reached 65%. Worms grown on 500mM NaCl plates contained Ub foci in 56% of nuclei (Figure 7 and 8). 6 hours was chosen to be used for further testing. Salt stress did have an effect on the morphology of the worm. As the NaCl concentration increased, worms were less responsive to touch and did not move normally. Additionally, the worms shrunk in length and increased in width. The GFP control strain did not contain any nuclear foci when stress was induced, but morphological changes similar to salt stressed worms did occur.

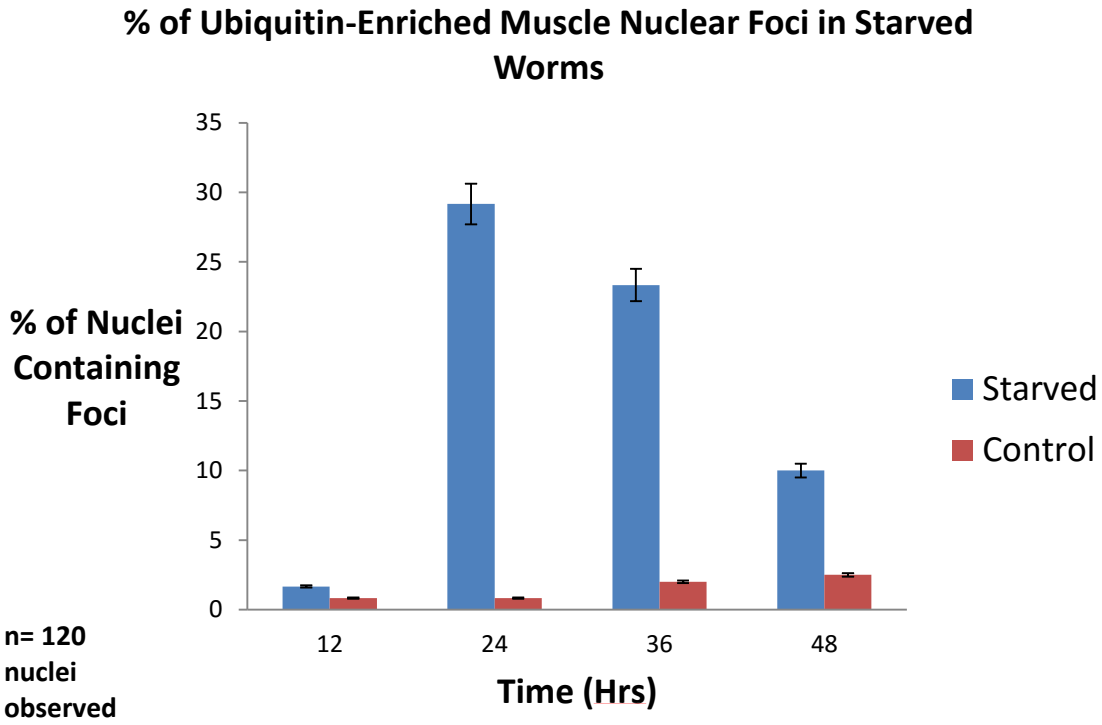

Figure 6. Foci are Observed in Starved Muscle Nuclei. 3 day adult worms were grown on starvation plates lacking *OP50 E. coli* and observed using confocal microscopy. 40 nuclei were observed in each of three separate experiments for a total of 120 nuclei observed. A total of 20 worms were observed over the course of the three experiments. Error bars represent standard error.

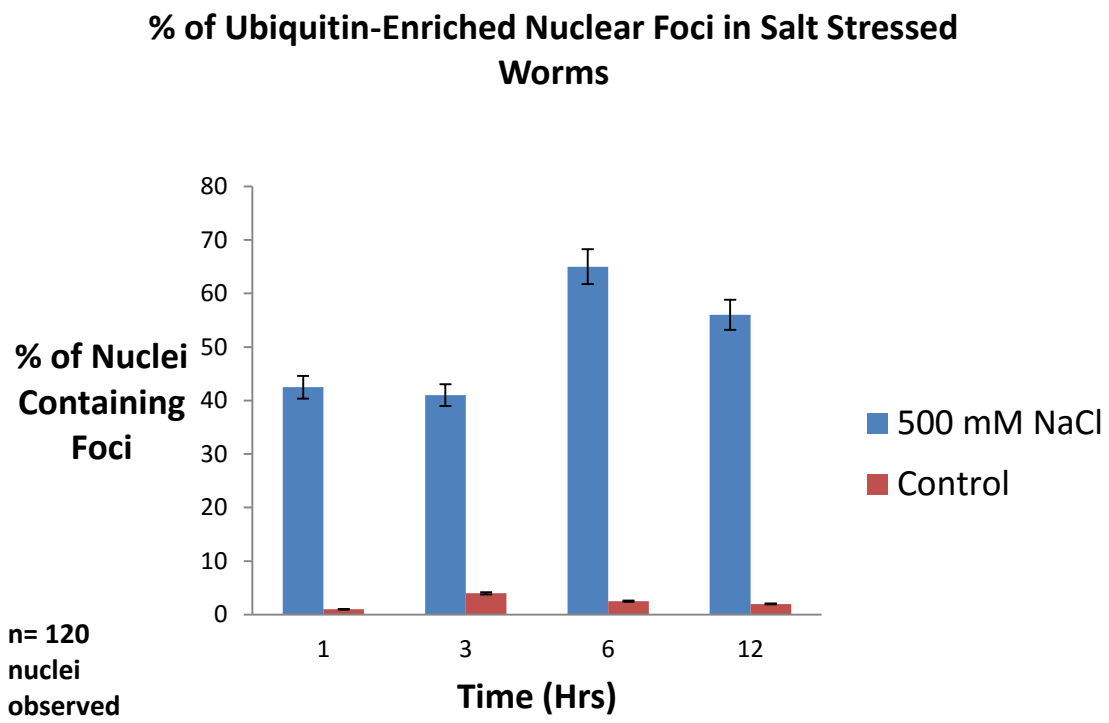

Figure 7. Foci are Observed in Salt Stressed Muscle Cells. 3 day adult worms were placed on NGM plates containing 500mM NaCl for desired time and observed using confocal microscopy. 40 nuclei were observed in each of three separate experiments for a total of 120 nuclei observed. A total of 20 worms were observed over the course of the three experiments. Error bars represent standard error.

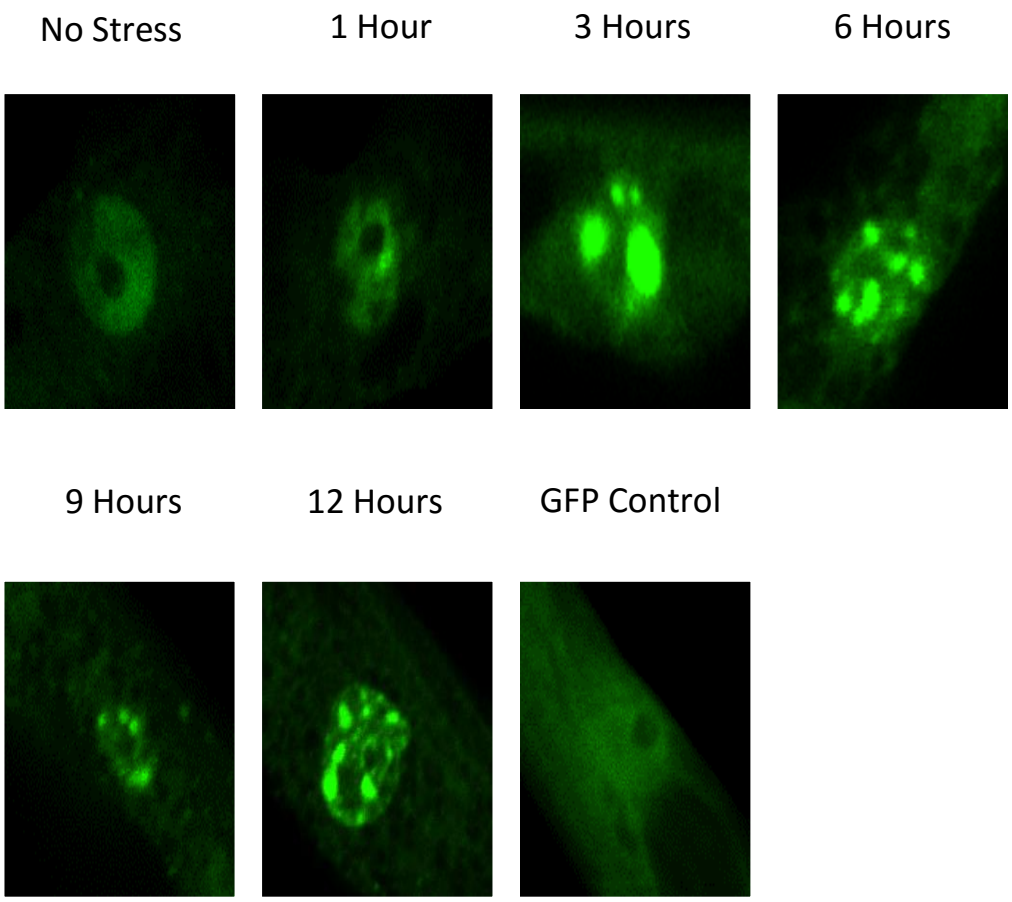

Figure 8. Foci Formation in Salt Stressed Muscle Nuclei of 3 day Adults. Worms were observed using 60X magnification on a Nikon confocal microscope.

Heat and Cold Shock Stress Does Not Result in Nuclear Foci

After noticing that starvation and osmotic stress resulted in the formation of nuclear foci, heat and cold shock stress experiments were performed Morton and Lamitina, 2013, determined that *C. elegans* subjected to elevated temperatures caused HSF-1 foci to form in nuclei. To determine if Ub is also involved in heat stress, 3 day adults were subjected to 35° C for 20 minutes and immediately imaged to observe if any foci were observed. After observing no changes in 40 nuclei, the time of incubation was increased to one hour, however, no Ub foci were observed in 120 nuclei. The conditions were chosen to match the conditions in the original Morton and Lamitina experiment.

Additionally, cold shock experiments were performed on LN133 3 day adults to determine if Ub foci form in cold shocked *C. elegans.* Worms were subjected to 4° C for 30 minutes or 1 hour and immediately observed to determine if nuclear foci formed. Similar to the heat shock experiments, nuclear foci did not form in 120 nuclei observed. After determining that the temperature may have been too cold, additional tests were performed at 16° C at 30 minutes and 1 hour and neither condition caused nuclear foci to form in 120 nuclei observed.

Both heat and cold shock conditions did not seem to have a major effect on the behavior of worms. In both conditions, worms were moving normally and had comparable offspring.

Intestinal Tissues Exhibit Similar Responses to Stress

After determining that muscle cells exhibit Ub foci in the nucleus when subjected to specific stressors, experiments were carried out to determine if other tissues have similar responses to these stressors. Intestinal nuclei were chosen and subjected to both starvation and salt stress. Both experiments were carried out similarly to previous experiments. Results for the aging and salt stress experiments were similar in both cases, but the longer the intestinal cells were subjected to the conditions the harder results were to obtain due to autofluorescence in the gut. Additionally, one major difference between muscle and intestinal tissues was the amount of foci that formed due to stress. While muscle tissue typically had multiple foci with no apparent pattern, intestinal nuclei typically contained only one focus found near the nucleolus. (Figure 9) Intestinal Ub foci were also typically uniform in shape as compared to muscle Ub foci that had a variety of different sizes. Another difference that was surprising was that starved intestinal cells did not have any significant foci formation.

After observing the amount of nuclei that contained foci in aging intestinal tissues, it was determined that there was not a statistical difference when compared to muscle tissues. However, 7 and 10 day adults were not able to be observed due to autofluorescence in the gut and high background noise. 1 day adults had about 11% of nuclei that contained Ub foci. 3 day adults had 2% of nuclei containing Ub foci. (Figure 10)

After observing a slight increase of foci formation in starved 3 day adult muscle cells, expectations were that 3 day adult intestinal nuclei would experience a higher amount of nuclear foci when subjected to starvation. However, intestinal tissues subjected to starvation contained fewer nuclei with Ub foci. 12 and 24 hours of starvation only resulted in 7% and 9% respectively. 36 hours of starvation resulted in 4% of nuclei containing foci. Results could not be obtained for intestinal nuclei subjected to 48 hours due to high background noise due to autofluorescence in the gut. (Figure 11)

Salt stress experiments did yield similar results as compared to muscle cells. 1 and 3 hours of salt stress resulted in 42% and 46% of nuclei with Ub foci respectively. 6 and 12 hours of salt stress resulted in 72% and 69% respectively. (Figure 12) This suggests that similar mechanisms are responsible for muscle and intestine to respond to salt stress. Heat and cold shock stress experiments were also carried out on intestinal nuclei and yield no nuclei that contained foci.

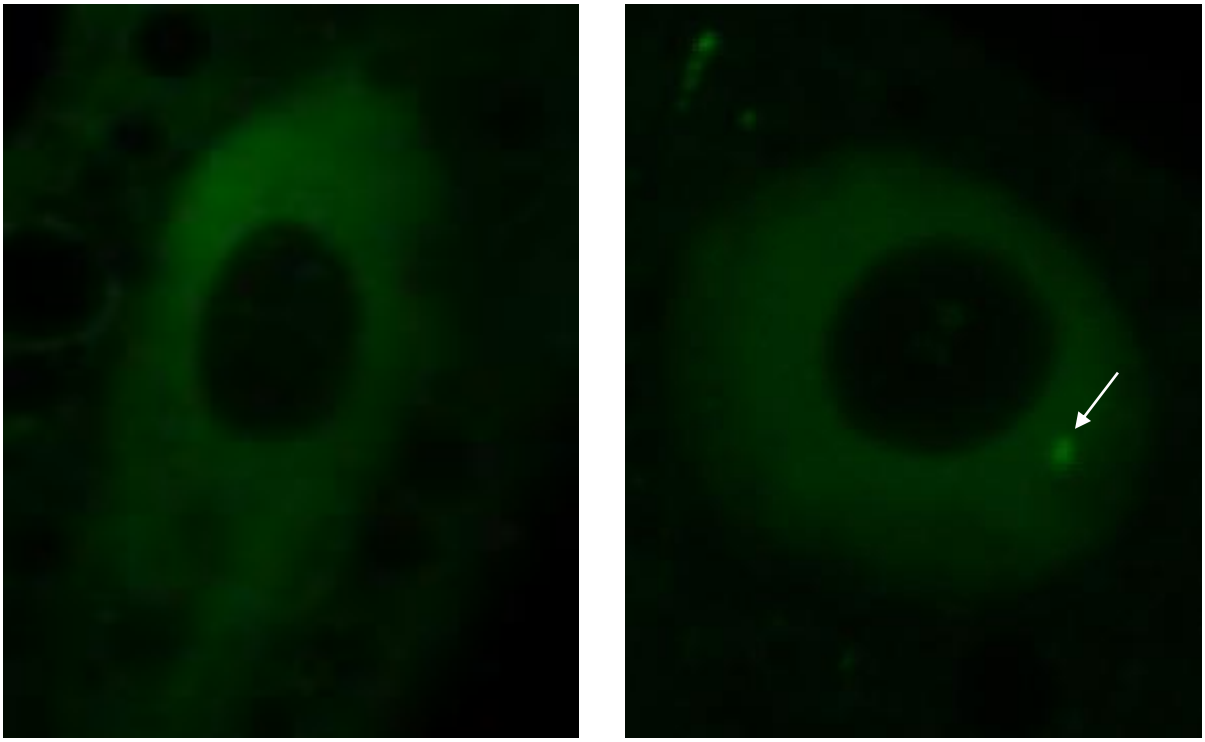

Figure 9. Foci Formation in Salt Stressed Intestinal Nuclei of 3 day Adults. 3 day adult worms were placed on NGM plates containing 500mM NaCl for 6 hours and observed using 60X magnification on a Zeiss confocal microscope. Arrow indicates nuclear Ub focus.

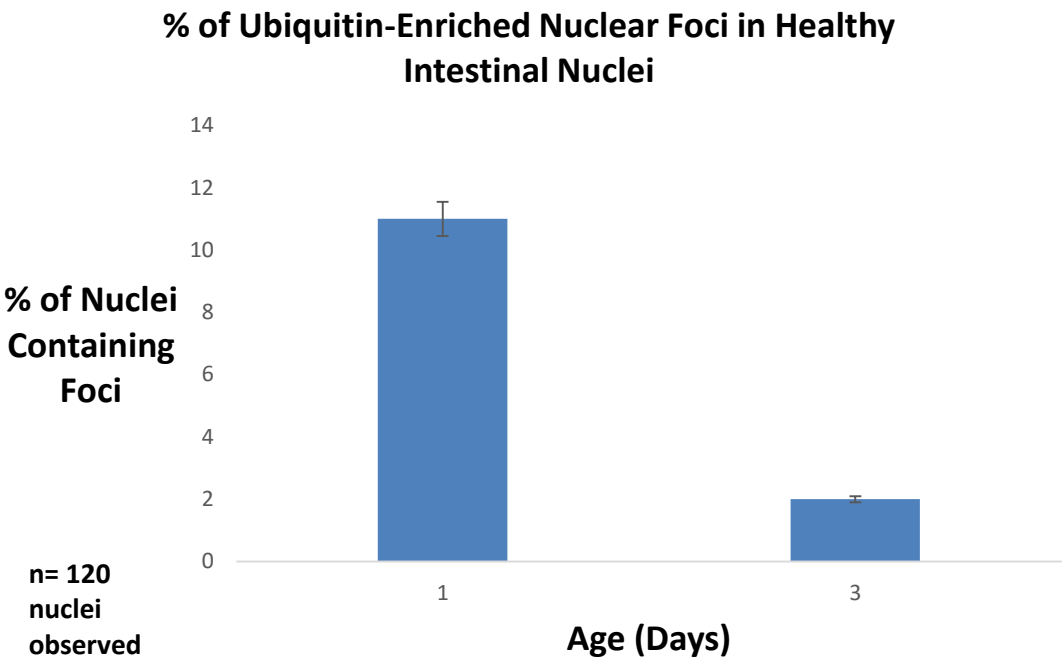

Figure 10. Foci are Observed in Aging Intestinal Nuclei. Graph showing the percent of muscle nuclei that express foci in varying ages of *C. elegans*. 40 nuclei were observed in each of three separate experiments for a total of 120 nuclei observed in. A total of 20 worms were observed over the course of the three experiments. Error bars represent standard error.

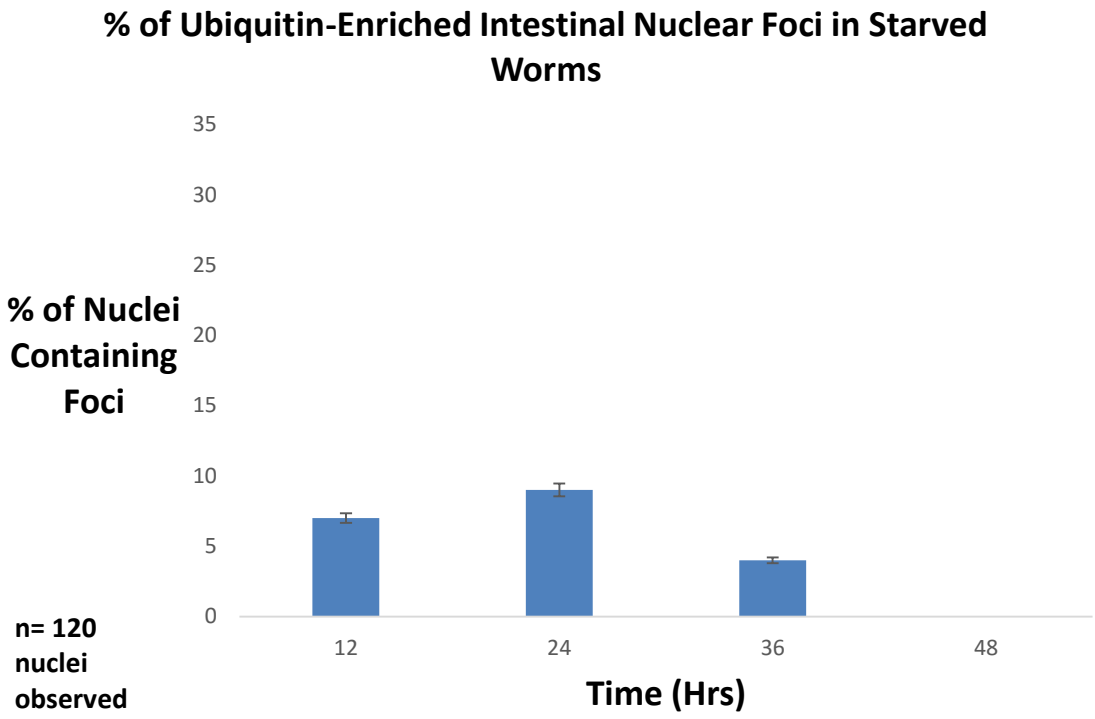

Figure 11. Foci are Observed in Starved Intestinal Nuclei. 3 day adult worms were grown on starvation plates lacking *OP50 E. coli* and observed using confocal microscopy. 40 nuclei were observed in each of three separate experiments for a total of 120 nuclei observed. A total of 20 worms were observed over the course of the three experiments. Error bars represent standard error.

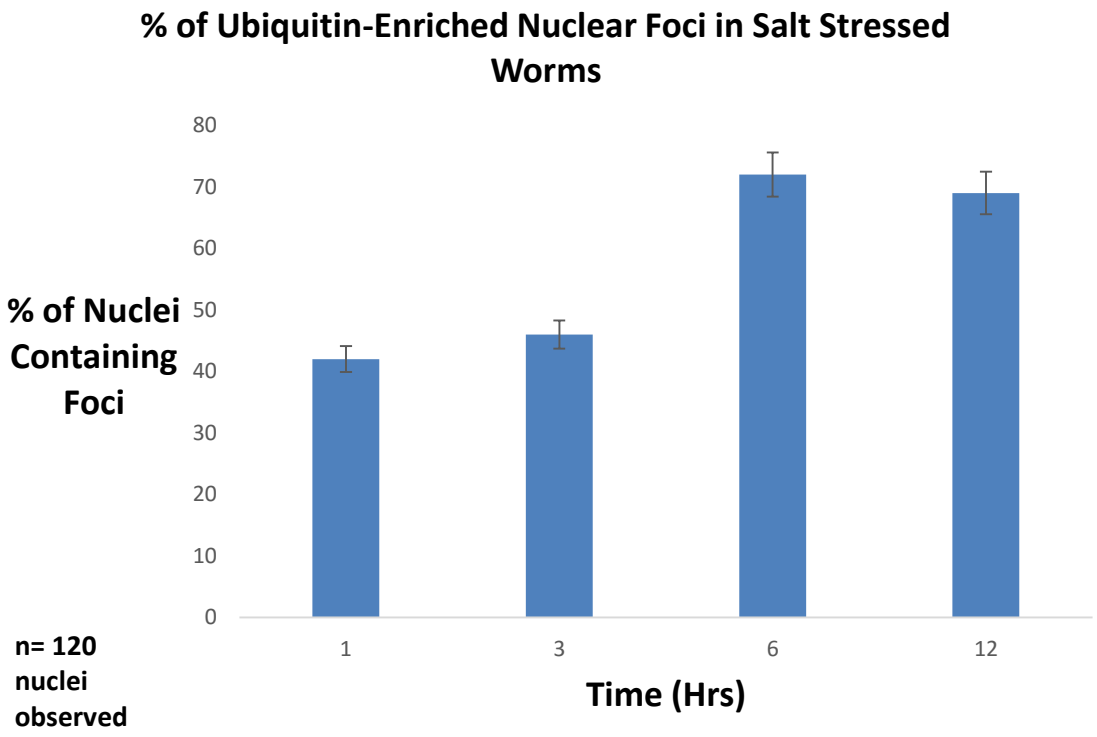

Figure 12. Foci are Observed in Salt Stressed Intestinal Nuclei. 3 day adult worms were placed on NGM plates containing 500mM NaCl for desired time and observed using confocal microscopy. 40 nuclei were observed in each of three separate experiments for a total of 120 nuclei observed. A total of 20 worms were observed over the course of the three experiments. Error bars represent standard error.

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CHAPTER IV:

DISCUSSION

Ub is a major regulator of cellular homeostasis. Ub is highly conserved and found in all tissue types in eukaryotes. The research presented here confirms that Ub is found to be diffuse in both muscle and intestinal cells. Furthermore, we have found that Ub can form nuclear foci as *C. elegans* age and when specific forms of stress are applied. However, not all forms of stress will induce nuclear foci to form. Limited studies have been conducted to study Ub in the nucleus. The UPS has been seen to function in the nucleus of yeast, but not in other organisms (Gardner et al., 2005). Ub foci formation has not been documented, but other foci have been documented to form when stress is induced.

Within the last two decades various nuclear structures have been thoroughly studied, but the current understanding of these nuclear structures are limited. It remains possible that these structure may be related to the Ub foci that we have observed when cells undergo aging, starvation, and salt stress. These structures include cajal bodies, promyelocytic leukemia (PML) bodies, and nuclear speckles (Cajal, 1903; Villagra et al., 2006; Boronenkov et al., 1998;). Cajal bodies are typically found in specific cell types such as neurons, embryonic, and tumor cells and have not been observed in muscle or intestinal nuclei (Ogg and Lamond, 2002). PML bodies are typically observed in diseased state cells, but have been observed in aging cells (Salamoni and Pandolfi, 2002). PML bodies have also been shown to aggregate with Ub in CAG repeat diseases (Yamada et al., 2001). Nuclear speckles are found in the interchromatin spaces

and exhibit a chain morphology (Thiry, 1995). It is hypothesized that nuclear speckles have some function of splicing but this hypothesis is highly debated (Lamond and Spector, 2003). The composition of these nuclear bodies typically contain ribonucleicproteins, but are localized in different tissues and areas of the nucleus. The major finding in our study is that nuclear foci containing Ub form. The Ub foci found in the nucleus provide an interesting direction in the nuclear field. Our finding provides an area of research to determine the relevance of Ub not only in the nucleus but also in the formation of nuclear foci. It remains possible that Ub foci and other nuclear bodies have a significant role in chromatin remodeling or other processes.

After determining that Ub has the tendency to form nuclear foci, we determined that aging caused this phenomenon. We did not expect to see this result and led us to determine the frequency of nuclei that contain foci. This result was not surprising due to the fact that aging has been shown to increase the frequency of damaged and misfolded proteins. While we are not confident that the foci formed during aging is due to misfolding of proteins, this result provides an avenue for further testing to determine the cause. It is more than likely that misfolding is occurring and degradation of the misfolded proteins would need to occur. We hypothesize that these proteins are being tagged for degradation, however, the substrates have not been identified. Our results are also consistent between tissues which suggests that a similar mechanism is occurring to tag

proteins. However, it remains unclear as to if the same proteins are being tagged in both tissues.

Moreover, we found that nuclear foci also form during starvation and salt stress. This confirmed our hypothesis that stress may be causing the formation of nuclear foci. Again the mechanism remains elusive as well as the substrates being targeted for ubiquitination. It also remains unclear whether foci formation during aging and stress are regulated by the same mechanism. We did observe that salt stress experiments did produce similar results. Conversely, we concluded that starvation experiments resulted in an unexpected outcome. Starvation in muscle nuclei had a major impact on nuclear foci formation while intestinal nuclei fewer nuclear foci. We hypothesize that intestinal cells may be one of the last tissues to be affected. Our reasoning is that intestinal cells would be needed to be functional and not be inhibited in the case that food became available. Another hypothesis is that the tissues contain different mechanisms to maintain starvation events.

More importantly, we observed that cells subjected to heat and cold shock stress did not contain nuclear foci. This result suggests that Ub functions differently when subjected to different stressors. Moreover, this suggests that different mechanisms are occurring to maintain homeostasis during stress. We hypothesized that both heat and cold shock would cause nuclear foci to form, but our hypothesis was disproven. This result has shifted our thinking of Ub’s role in stress. Nuclear stress bodies (NSBs) have been found to form when cells

undergo heat shock. The protein HSF-1 is found to form stress granules in the nuclei when the nuclei experience heat shock (Mahl et al., 1989; Sarge et al., 1993) The function of NSBs remains elusive but the current hypothesis is that NSBs are responsible for recruiting splicing and transcription factors which can control gene expression (Biamonti and Vourc'h, 2010). We find that the Ub foci observed are unlikely to be related to the NSBs formed due to heat shock because no Ub foci formed when subjected to heat shock.

Our research has provided an exciting future for Ub studies. Future experiments to determine the substrates of nuclear foci must be conducted. Results from these experiments may provide a better understanding of what is occurring during stress. Mass spectrometry techniques have grown to provide an avenue to gain insight into a variety of scientific issues. Mass spectrometry would provide and outlet to determine the substrates in aging and stressed cells. Attempts were made to extract Ub and its substrates from healthy, aged, and stressed cells for mass spectrometry analysis but we were not able to obtain a pure Ub sample. This information could provide answers into what substrates are being ubiquitinated. This may also provide an understanding if the same mechanisms are occurring in aging and stressed cells. We hypothesize that different substrates and mechanisms are occurring under each condition.

Another possible avenue of future research is to determine other stressors cause nuclear foci formation. If different mechanisms are in fact occurring due to different stressors, the understanding of Ub's broad role in cellular homeostasis

may be further understood. It would also be prudent to study stress effects on other tissues. Attempts to study the role of stress in hypodermal tissues were made but initial results were not successful due to the GFP construct not being expressed. Understanding the role of stress in other tissues may provide great detail of Ub's role in stress at an organismal level. Further attempts in other organisms must also be conducted to ensure that this phenomenon is not a *C. elegans* specific event.

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